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CHOLINE INCORPORATION INTO LECITHIN IN RESPONSE TO INSULIN OR DEXAMETHASONE IN HOMOGENEOUS CELL CULTURES OF RAT LUNG EPITHELIAL CELLS AND FIBROBLASTS

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SUMMARY

Labeled choline incorporation into adult rat lung type II alveolar epithelial cells and adult rat lung fibroblasts in monolayer culture was determined after incubation with insulin (Ins) 10 µg/ml, Dexamethasone (Dex) 10^{-6}M, or no drug (ND). Incubation periods were 1, 3, 4, and 5 hours. The lecithin (phosphatidyl choline - PC) recovered was separated into disaturated phosphatidyl choline (DSPC) and unsaturated phosphatidyl choline (USPC). Results expressed as specific activity per hour (see Table) indicate that the incorporation of choline into PC and USPC was greater in fibroblasts (F) than in epithelial cells (E) whether ND, Dex or Ins was present. For incorporation into DSPC, there was no difference between E and F whether ND, Dex or Ins was present. There was significant increase in choline incorporation into PC or USPC for both cell types when Ins was present, whereas there was no difference for either cell type when Dex was present. Insulin significantly increased choline incorporation into DSPC in E cells only. Dex was no different from ND in DSPC incorporation in either cell type. We attribute the greater lecithin synthesis of the F cells to a more rapid increase in cellular structural lipids in the fibroblast cell. Dex had no effect on either cell type possibly from the short-term exposure or possibly because the effect of dexamethasone on alveolar epithelial cells is mediated by product(s) from other lung cells, and thus requires a mixed cell culture to have its effect. We suggest that further study of isolated homogeneous cell lines will not be fruitful in the evaluation of mechanisms of acceleration of lung maturation.

SPECULATION

Isolated alveolar epithelial cells show a relatively greater increase in DSPC synthesis than do isolated fibroblasts in response to insulin, while neither cell type responds to short term glucocorticoid treatment. Since other investigators using mixed lung cell cultures have shown increased DSPC in response to insulin and even greater response with glucocorticoid treatment, it is likely that the presence of fibroblasts or other pulmonary cells is required for the alveolar epithelial cell to increase DSPC production in response to glucocorticoids.

INTRODUCTION

Previous in vitro studies of the regulation and control of pulmonary lecithin synthesis have been based upon experiments using mixed lung cell cultures(8-12). One of the problems of this approach is that lung fibroblasts replicate much faster in culture than epithelial cells(3).Thus, in mixed lung cell culture the epithelial cells are often overgrown by fibroblasts resulting in a culture population that is no longer representative of the lung. Some investigators have questioned whether the effects of drugs and manipulations on these mixed cell cultures' lecithin synthesis is due to the effect on the alveolar epithelial cells alone(10). To address this question we used separate, homogeneous cell lines to evaluate the effect of dexamethasone and insulin upon lecithin synthesis.

MATERIALS AND METHODS

Established, characterized lines of adult rat lung type II alveolar epithelial cells (population doubling #32) and adult rat lung fibroblasts were simultaneously maintained in separate monolayer cell culture at the W. Alton Jones Cell Science Center, Lake Placid, New York using T75 flasks (Falcon, CA) containing Ham's F12K culture medium (GIBCO, NY), supplemented with 10% fetal bovine serum (GIBCO, NY), aqueous Penicillin G (Parke-Davis, MI) and Kanamycin (GIBCO, NY). After adding ^{3}H-choline chloride (New England Nuclear, MA) in a sterile fashion to F12K culture medium and mixing it well to attain a fluid concentration of 0.3 µCi/ml, three aliquots of this labeled medium were separated into sterile containers. To the first, porcine regular insulin (Lilly, IN) was added to make a final concentration of 10 µg/ml. To the second, dexamethasone sodium phosphate, U.S.P. (Organon, NJ) was added to make a final concentration of 10^{-6}M. No drug was added to the third aliquot, which served as a control.

Three days after passage, the original growth medium was discarded and 20 ml of ^{3}H-choline labeled medium (with or without drug) warmed to 37°C was added to each T75 flask. Each flask was immediately placed in a 37°C CO_2 incubator (Wedco, MD). Incubation periods were exactly 1, 3, 4, and 5 hours. Duplicate samples for each cell type, drug type, and incubation time were utilized. At the end of the incubation, the labeled medium was discarded and the cells rapidly washed three times with warm saline to remove the majority of labeled medium coating the cells. Five ml of 37°C 0.25% trypsin (GIBCO, NY) in Hanks' balanced salt solution (GIBCO, NY) was added. The cells were harvested after 15 minutes of trypsinization. Chilled non-labeled medium was added in equal amount to inactivate the trypsin. The cells were dispersed and washed three times with cold saline to remove labeled medium adherent to the cell surface. A small aliquot was removed for cell count. The cells were disrupted and frozen in 0.5 ml triple-glass-distilled water.

After thawing, the lipids were extracted using a chloroform-methanol 2:1 (V/V) mixture. 0.001 µCi of ^{14}C-labeled dipalmitoyl phosphatidyl choline (^{14}C-DPPC) was added to the total lipid fraction as a tracer for disaturated lecithin recovery. This amount of tracer was sufficient to give enough counts to trace the ^{14}C-DPPC with minimal spillover into the tritium channel. Each total lipid sample was dissolved in chloroform and spotted on an activated silica gel G plate (Applied Science Laboratories, Inc., PA). The phospholipids were separated using two-dimensional thin layer chromatography (TLC). The first dimension consisted of chloroform-methanol-7N ammonia (65:20:4) and was run twice. The second dimension was chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5). Each plate was dried under nitrogen for 15 minutes between dimensions. The spots were visualized using dichlorofluorescein in ethanol and the lecithin spot was removed. After washing with solvent and centrifugation, the silica was discarded. The lecithin was dissolved in chloroform and an aliquot was removed for scintillation spectrometry using a Packard Tricarb Scintillation Spectrometer model 3320 and for phosphorous determination via the method of Hess and Derr(4).

The lecithin (phosphatidyl choline - PC) was separated into disaturated phosphatidyl choline (DSPC) and unsaturated phosphatidyl choline (USPC) using a modification of the method of Mason, et al(5). After the lipid was reacted with osmium tetroxide it was evaporated under nitrogen and redissolved in chloroform-methanol 20:1 (V/V). It was then spotted on an activated silica gel G plate and single dimension thin layer chromatography was performed using a solvent system of chloroform-acetone-methanol-acetic acid-water 50:20:10:10:5 (V/V). After drying the plate under nitrogen, the DSL was visualized using dichlorofluorescein in ethanol. The DSL and USL fractions were removed, washed in solvent, and the silica discarded. After evaporation under nitrogen, the samples were redissolved in chloroform and aliquots were taken for scintillation spectrometry and phosphorous determination. A tritium standard and a ^{14}C-DPPC standard sample were counted along with the study samples. Correction was made for the percent spillover from the ^{14}C channel to the tritium channel and vice versa for each study sample prior to analyzing the results.

RESULTS

The results are expressed as specific activity per hour (CPM/nMole phosphorous/hr) which eliminates differences in cell number per flask (range 0.9 x 10^{6} - 7.8 x 10^{6} cells/flask) and differences in incubation time. The incorporation of ^{3}H-choline into all PC fractions was linear throughout the time period used, which is in agreement with previous work by Smith and Torday(10). Recovery of PC was greater than 90%. Recovery of ^{14}C-DPPC was 58%. Gas chromatographic analysis of DSPC obtained using the osmium tetroxide method reveals that both cell types contain 97% saturated fatty acids, the majority being palmitate (75-78%) (6).

Using analysis of variance, the incorporation of choline into PC and USPC was significantly greater in fibroblasts (F) than epithelial cells (E) in every instance, whether no drug (ND) ($p < .001$), Dexamethasone (Dex) ($p < .05$) or Insulin (Ins) ($p < .02$) was present (see Table). There was no difference between E and F whether ND, Dex or Ins was present with regard to incorporation into DSPC.

Evaluating PC and USPC, there was significant ($p < .001$) increase in choline incorporated when Ins was present in the medium for both E and F cells. There was no significant difference in choline incorporation into PC or USPC for either cell type when Dex was present as compared to ND.

Evaluating the DSPC fraction, Ins significantly ($p < .05$) increased choline incorporation in E cells only. Dex was not different from ND in DSPC incorporation in either cell type.

DISCUSSION

Unlike other studies using cell culture, adult rather than fetal cells were utilized because all morphologic characterizations and enzymatic studies have been documented on the adult type II alveolar cell(2,3). It is possible that studies performed upon homogeneous fetal type II alveolar cells may have given different results. However, we did confirm stimulation of lecithin synthesis in both adult cell types when insulin was added, a phenomenon that has been reported by Smith using mixed cell cultures of fetal rabbit lung(9). The positive effect of insulin upon lecithin synthesis may be due to the general growth promoting effect of insulin.

Because L-2 cells do not remain diploid in culture for more than 35-40 population doublings (unpublished data by author WHJD) at which time they dedifferentiate and lose their lamellar bodies, we used cells of passage 32. Karyotyping confirmed the cells to be diploid. Ultrastructural examination confirmed that the epithelial cells contained lamellar bodies characteristic of type II pneumatocytes. Because nutritional stress and transformation are more likely to occur with confluency of these cells, we chose to perform our experiments with the cells in a subconfluent state. It is possible that we might have found a greater increase in DSPC production in the E cells had they been confluent and stimulated to make product, presumably DSPC, rather than to divide and fill the empty spaces of the flask surface. We attribute the greater lecithin synthesis of the fibroblastic cells to a more rapid increase in cellular structural lipids occurring with the more rapid growth of the fibroblast cell.

Smith, et al reported that cortisol has a positive effect on choline incorporation during a 6-hour incubation period(10). We chose to test dexamethasone because it is one of the glucocorticoids administered successfully to pregnant women to accelerate maturation of the fetal surfactant system(13). Ballard has demonstrated dexamethasone receptors in the alveolar epithelial cells used in this experimental protocol(1). Previous reports have shown a positive effect of dexamethasone when it was present in the culture medium with the cells for several days before labeled choline incorporation was measured(10). The dose of dexamethasone was determined after reviewing the literature and after studying the effect of incubating both cell types in medium containing 10^{-8}M dexamethasone. We cannot eliminate the possibility that the dose was inhibitory to lecithin production. Smith and Torday demonstrated enhancement of choline incorporation with 5.5 x 10^{-8}M dexamethasone, thus we are confident that the dosage used was not too low to demonstrate a positive effect(10).

It is likely that the effect of dexamethasone on alveolar epithelial cells is mediated by product from other lung cells and requires a mixed cell culture to have its effect. Indeed, recent reports from Smith, et al indicate that lung maturation in the fetal rat is accelerated by injection of a fibroblast-pneumonocyte factor produced by the fetal lung fibroblasts(7,8). Thus, we suspect that further study of isolated, homogeneous cell lines will not be fruitful in the evaluation of mechanisms of acceleration of lung maturation.

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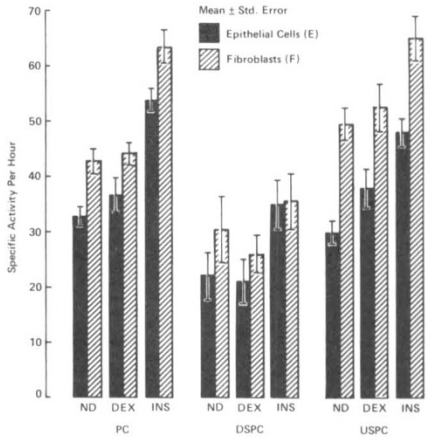
TABLE I



Effect of no drug (ND), dexamethasone (Dex) or insulin (Ins) upon production of phosphatidyl choline (PC), disaturated phosphatidyl choline (DSPC) and unsaturated phosphatidyl choline (USPC) in adult rat lung epithelial cells (E) and adult rat lung fibroblasts (F).

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